

New chloride-activated aminopeptidase from human erythrocytes

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A new Cl^- -activated aminopeptidase was purified from the cytosol of human erythrocytes as a single chain protein of an approx. M_r of 70 000 and pI of 5.1. The enzyme hydrolysed 2-naphthylamides of aliphatic, aromatic and basic L-amino acids, with a preference for the alanyl residue. It also hydrolysed di-, tri-, and some hydrophobic tetrapeptides. The inhibitors were bestatin, amastatin, Co^{2+} , Zn^{2+} , Mn^{2+} , 4-hydroxymercuribenzoate and 1,10-phenanthroline. The activity of the enzyme, inhibited by 4-hydroxymercuribenzoate, was partially restored by the addition of sulfhydryl compounds.

The presence of 0.2 M Cl^- (Br^- , F^-) caused a several-fold increase in the isolated aminopeptidase activity.

Aminopeptidase; Alanyl aminopeptidase; Halide activation; (Erythrocyte, Human)

1. INTRODUCTION

Most of the mammalian aminopeptidases were recognized as metal-activated enzymes [1], whereas activation by halide anions was observed only with the soluble arginyl and cystinyl aminopeptidase [2–8].

We have observed that the cytosol of human erythrocytes contains three proteolytic enzymes which cleave the aminopeptidase substrate, Arg-2NA. One of them was isolated and characterized as an aminopeptidase of broad specificity, activated by Co^{2+} [9]. The second one most probably corresponds to the enzyme identified as the chloride-activated arginyl aminopeptidase [3,10,11]. The present study describes the purification and characterization of the third Arg-2NA hydrolysing enzyme derived from the cytosol of human erythrocytes, which was shown to be a new alanyl aminopeptidase, markedly activated by chloride ions.

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Abbreviations: 2NA, 2-naphthylamide; 4Nan, 4-nitroanilide; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing

2. MATERIALS AND METHODS

2.1. Enzyme purification

Fresh human erythrocytes, washed and free of white cells, were lysed by the addition of 5.5 vols of 5 mM sodium phosphate, pH 6.8. Membranes were removed by centrifugation (1 h, $20000 \times g$) and cytosol (940 ml) applied (120 ml/h) to a DEAE-cellulose column (5×13 cm) equilibrated with a 10 mM sodium phosphate buffer, pH 6.8. The column was rinsed with the starting buffer, and then with 10 bed vols of a linear NaCl gradient (0.0–0.14 M) in the same buffer. The new Cl^- -activated aminopeptidase was eluted between 0.045 and 0.055 M NaCl, dialysed and rechromatographed on the DEAE-cellulose column (0.9×18 cm) using a 10 mM sodium phosphate buffer, pH 6.9, with 1 mM 2-mercaptoethanol, and 0.0–0.14 M NaCl gradient. The last purification step was gel filtration on a Sephacryl S-200, superfine, column (2.5×88 cm) in the same buffer containing 0.2 M NaCl. Enzyme preparation was concentrated by ultrafiltration and stored in 40% glycerol at -10°C .

2.2. Enzyme and protein assay

Aminopeptidase activity was determined in a reaction mixture containing 0.089 mM Ala-2NA or another amino acid-2NA (Serva), 25 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl and an appropriate amount of enzyme, as previously described [12]. Activity of the purified aminopeptidase was also determined with Ala-4Nan (0.5 M) by continuous recording of liberated 4-nitroaniline as A_{405} [13]. The unit of activity was defined as the amount of enzyme which splits 1 μM of substrate per min. Proteolytic activity was assayed at pH 4.0, 7.5 and 8.6 by overnight incubation of 0.1% Azocoll (Sigma) with 3.5 $\mu\text{g}/\text{ml}$ of the enzyme.

Proteins were monitored as absorbance at 280 nm or determined by the method of Bradford [14] using bovine serum albumin as a standard.

2.3. Degradation of peptides

Aminopeptidase (2.5 µg/ml) was incubated with 0.41 mM peptide in 50 mM Tris-HCl, pH 7.5, for 1 h at 37°C. Aliquots of 5 µl were analysed by thin-layer chromatography [12] and intensities of spots compared visually with those of standards.

2.4. Electrophoresis and isoelectric focusing

These were performed by the PhastSystem (Pharmacia, Sweden) using PhastGel gradient 8–25 plates and PhastGel IEF 3–9 plates, respectively. For the SDS-PAGE the samples were treated with 2.5% SDS and 5% mercaptoethanol for 5 min at 100°C.

2.5. Relative molecular mass

This was determined by SDS-PAGE, and by gel filtration on a Bio-Gel P-100 (Bio-Rad) column (1.6 × 86 cm) in 50 mM Tris-HCl buffer, pH 7.9, with 0.5 M KCl, at a flow rate of 20 ml/h. Low M_r calibration kits from Pharmacia were used.

3. RESULTS

3.1. Enzyme purification

The isolation of Cl^- -activated aminopeptidase from human erythrocytes cytosol is summarized in table 1. By the described procedure 23000-fold purification was achieved, whereas the yield is most probably higher than 17%, since the broad specificity aminopeptidase also contributes to the hydrolysis of Ala-2NA in the cytosol. The isolated enzyme was homogeneous by PAGE and IEF. Determination of aminopeptidase activity performed on gels after PAGE, revealed that the protein band coincided with the area of hydrolytic activity.

3.2. Enzyme characterization

The relative molecular mass of the isolated aminopeptidase determined by SDS-PAGE and by gel filtration was about 65000 and 72000, respectively. No subunits were detected. Its isoelectric point as determined by IEF was at pH 5.1. The enzyme did not lose activity for several weeks in 40% glycerol at pH 7.0 and 4°C. Addition of 2-mercaptoethanol was beneficial to enzyme stability.

The optimal pH for the hydrolysis of Ala-2NA in the presence of chloride ions was pH 7.6. The Cl^- -activated aminopeptidase was inhibited by bestatin and amastatin but was resistant to leupeptin and puromycin. Inhibition was also pronounced

Table 1

Purification of Cl^- -activated aminopeptidase from human erythrocytes

Purification step	Volume (ml)	Protein (mg/ml)	Spec. act. (mU/mg)	Yield (%)
Cytosol	944.0	64.40	0.066	100
I DEAE-cellulose, ultrafiltration	54.5	0.25	64.72	22
II DEAE-cellulose	6.5	0.52	250.04	21
Sephacryl S-200	16.5	0.028	1513.30	17

ed in the presence of 4-hydroxymercuribenzoate and 1,10-phenanthroline. EDTA was less potent and inhibited the enzyme only at a concentration of 10^{-2} M, as lower concentrations enhanced the enzyme activity. The inactivation of the enzyme by 4-hydroxymercuribenzoate was partially reversed by the addition of thiol compounds. Thus 4 mM 2-mercaptoethanol or 2 mM dithiothreitol restored 34% of the aminopeptidase activity. Activation by thiol agents was observed as well, but with the enzyme which was extensively dialysed against the buffer without 2-mercaptoethanol. Metal ions, Co^{2+} , Zn^{2+} and Mn^{2+} already at a concentration of 0.01 mM were inhibitory, whereas 1 mM Ca^{2+} was without effect. The hydrolysis rate of Ala-4Nan was also diminished in the presence of 0.1 mM L-amino acids having hydrophobic side chains.

3.3. The effect of halide ions

The isolated aminopeptidase had higher specific activity in Tris-HCl buffer than in the sodium phosphate buffer of the same pH and similar ionic strength. It was shown that this difference can be attributed to the Cl^- activation of the enzyme (fig.1A). The effect of Cl^- was maximal around 0.2 M NaCl and it was pronounced with Ala-, Leu- and Phe-2NA as substrates. Similar activation was observed upon the addition of KBr or NaF to the reaction mixture (fig.1B). KI had an inhibitory effect. No increase in the activity occurred when the concentration of phosphate was raised from 25 to 100 mM.

3.4. Enzyme specificity

The measurement of hydrolysis rates of different amino acid-2NA by isolated Cl^- -activated

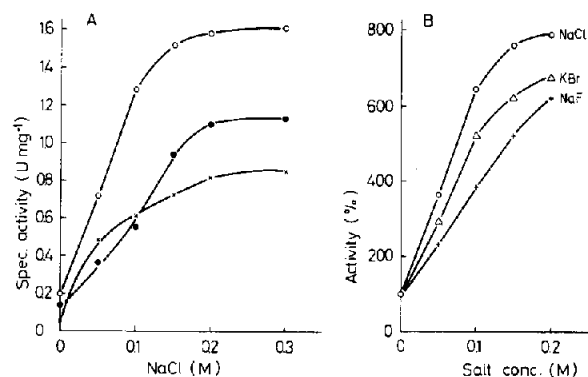


Fig.1. (A) Effect of halide ions on the activity of aminopeptidase from human erythrocytes in 25 mM sodium-phosphate buffer, pH 7.5. (A) Effect of NaCl on the rate of hydrolysis of (○) Ala-2NA, (●) Phe-2NA and (×) Leu-2NA. (B) Effect of halide ions on the rate of hydrolysis of Ala-2NA.

aminopeptidase revealed that the enzyme prefers Ala-2NA to the other tested substrates and does not cleave Glu-, Hyp- and D-Ala-2NA (table 2). It was shown that Leu-, Pro- and Arg-2NA behave as competing inhibitors of Ala-4Nan hydrolysis. The enzyme did not degrade proteinase substrate Azocoll or *N*-acetyl-Ala₃-4Nan, a substrate of *N*-acetylalanine aminopeptidase [15].

The action of isolated aminopeptidase on different peptides was also tested. Under the experimental conditions tripeptides, Ala-Ala-Ala, Leu-Gly-Gly and Phe-Gly-Gly, were completely degraded. A significant degradation of dipeptides, Arg-Phe, Leu-Val, Val-Val, Val-Tyr, Phe-Ala, Phe-Phe, and tetrapeptides, Leu-Leu-Val-Tyr and

Table 2

Hydrolysis of amino acid-2-naphthylamides by Cl⁻-activated aminopeptidase from human erythrocytes

Substrate	Relative rate (%)	Substrate	Relative rate (%)
Ala-2NA	100	His-2NA	3
Phe-2NA	51	Ser-2NA	2
Leu-2NA	48	Gly-2NA	1
Pro-2NA	27	α -Glu-2NA	0
Met-2NA	23	Hyp-2NA	0
Arg-2NA	18	D-Ala-2NA	0
Val-2NA	15	Ala-Ala-2NA	9
Tyr-2NA	15	Ala-Phe-2NA	5
Lys-2NA	8		

Phe₄ was found too. Tuftsin, Leu-enkephalin, bradykinin, and angiotensins I, II and III were not hydrolysed at all.

4. DISCUSSION

Intracellular aminopeptidases are classified on the basis of their substrate specificity and subcellular localization [1]. By its lack of specificity toward a particular amino acid-2NA and the preference for alanyl derivative, the aminopeptidase from human erythrocytes, described in this work, resembles the soluble alanyl aminopeptidase from human liver [13]. However, these two enzymes differ in *M_r*, in pH optimum, in response to Co²⁺ and puromycin, and in that which is most important in the susceptibility to Cl⁻ activation. The specificity of the erythrocyte aminopeptidase is also similar to that of aminopeptidase from human skeletal muscle [16], but this enzyme is activated by Ca²⁺ and not by Cl⁻.

The halide ions stimulated soluble arginyl aminopeptidases isolated from several human tissues [2-7], and the cystinyl aminopeptidase from skeletal muscle [8], clearly have different specificities towards amino acid naphthylamides, than the presently described enzyme. Also, endopeptidase action found for arginyl aminopeptidase [4,5,7] was not observed with the new Cl⁻-activated enzyme from human erythrocytes. Following the criteria applied to other aminopeptidases the enzyme isolated from human erythrocytes can be named Cl⁻-activated alanyl aminopeptidase.

However, newly isolated aminopeptidase has a number of properties in common with other Cl⁻-activated aminopeptidases. These are: the similar activation by halide ions, the presence of SH groups responsible for enzyme activity, inhibition by bestatin and not by puromycin, inhibition by metal cations and by 1,10-phenanthroline, with low sensitivity to EDTA at the same time. Arginyl aminopeptidase from skeletal muscle and brain, muscle cystinyl aminopeptidase, and new aminopeptidase from erythrocytes also have similar *M_r* and monomeric structure. Thus Cl⁻-activated aminopeptidases might belong to the same class of aminopeptidases, defined by the criteria not used in classification of these enzymes so far.

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